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Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

The role of the antennae during courtship behaviour in the parasitic wasp *Trichopria drosophilae* / R. ROMANI; M. ROSI; N. ISIDORO; F. BIN. - In: JOURNAL OF EXPERIMENTAL BIOLOGY. - ISSN 0022-0949. - STAMPA. - 211(2008), pp. 2486-2491. [10.1242/jeb013177]

Availability:

This version is available at: 2158/351052 since:

Published version:

DOI: 10.1242/jeb013177

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The role of the antennae during courtship behaviour in the parasitic wasp *Trichopria drosophilae*

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Accepted 19 May 2008

SUMMARY

We have studied the courtship behaviour of *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae), a pupal parasitoid of the common fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), to understand the role of the antennae. Virgin pairs of the parasitoid perform an intense and stereotyped antennal courtship, which leads to copulation. During antennation, the two male fourth antennomeres come into contact with the two apical female antennomeres, and thus the secretion produced by the sex pheromone gland is spread onto the female receptors. By preventing the transfer of the courtship pheromone from male to female antennae, mating was inhibited. Moreover, selective ablation of single antennae demonstrated that the courtship pheromone acts on contact. When antennae of both sexes were partially removed (ablation at the same side, i.e. right or left) courtship was successful and copulation occurred. In contrast, in the case of antennal ablation at opposite sides, courtship failed despite the short distance between secretion and receptors. These results confirm the hypothesis that *T. drosophilae* male antennal glands are the release site of a contact courtship pheromone, playing a key role in mating behaviour. The occurrence of male antennal glands in Hymenoptera and other insect orders is discussed.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/211/15/2486/DC1>

Key words: insect, mating behaviour, parasitoids, *Drosophila melanogaster*, antennal glands, sex pheromone.

INTRODUCTION

Courtship and mating behaviour are among the most important tasks carried out by animals during sexual reproduction (Cade, 1985). In insects, the largest class of living animals, mating behaviour is mediated by different kinds of signals between the male and female. Signals involved in insect sexual communication vary considerably. In some groups the emission of vibrations can be essential (Virant-Dobertlet and Čokl, 2004, and references therein). Visual cues are well known, i.e. the mating behaviour performed by some *Drosophila* (Speith, 1974). Besides these few cases, chemical cues, particularly sex pheromones, have a great relevance, being the most common signals used by insects during sexual communication. In parasitic wasps belonging to the order Hymenoptera, sex pheromones have been described in a few species (Vinson, 1972; Eller et al., 1984), and there are a few papers dealing with the identification of the sex pheromone gland (for a review, see Kainoh et al., 1999). These glands have been described in different regions of the female body [i.e. Dufour's gland (Vinson et al., 1978; Simser and Coppel, 1980; Syvertsen et al., 1995); base of the second valvifer: (Tagawa, 1977; Tagawa, 1983; Weseloh, 1980; Field and Keller, 1994)] and the role of the secretion has been related to long–middle range attraction or trail following of the conspecific. In parasitic wasps, mating behaviour can be defined as a sequence of stereotyped and species-specific behavioural steps, which can be summarized as follows.

(1) Mate location at long, middle or short range, often mediated by sex pheromones (Ridsdill Smith, 1970; Lewis et al., 1971;

Vinson, 1972; Robacker et al., 1976; Robacker and Hendry, 1977; Alcock, 1981; Fauvergue et al., 1995).

(2) Mate recognition, occurring after contact between individuals. In this step, a more or less complicated 'courtship behaviour' ritual occurs (Barras, 1960; van den Assem, 1969; van den Assem and Putters, 1980; van den Assem and Jachmann, 1982; Dahms, 1973; Gordh and DeBach, 1978; Viggiani, 1985; Isidoro et al., 1999).

(3) Copulation, during which the male transfers its spermatozoa to the female.

(4) Post-copulation, mostly characterized by grooming behaviour.

During courtship, intense and sometimes complicated antennal movements are performed by the male, often accompanied by antennal contact between sexes (Goodpasture, 1975; van den Assem et al., 1982; Takahashi and Sugai, 1982). Following evidence from these behavioural studies, Dahms (Dahms, 1984) reported the first case of antennal glands in Hymenoptera, possibly related to courtship behaviour. During the last 20 years, several cases of male antennal glands have been reported in different Hymenoptera families (Bin and Vinson, 1986; Isidoro and Bin, 1995; Isidoro et al., 1996; Bin et al., 1999; Romani et al., 1999; Guerrieri et al., 2001). In this paper, the mating behaviour of *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae), a pupal parasitoid of the fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), was studied.

Behavioural experiments were carried out to define the role of male antennal glands during courtship, as a possible source of a male-produced sex recognition pheromone. In *T. drosophilae*, a

previous study (Sacchetti et al., 1999) revealed the presence of male antennal glands. Because of the relatively large body size of individuals and their slow movements during courtship, and also because of the presence of a single male antennomere (the fourth, A4, counted from the scape) bearing the glands, *T. drosophilae* makes an excellent model for this kind of behavioural study.

MATERIALS AND METHODS

Insects

Both *D. melanogaster* and *T. drosophilae* colonies were obtained from the 'Laboratoire de Biométrie, Génétique et Biologie des Populations', Claude Bernard University, Lyon (France), and maintained in a laboratory (temperature, 25±2°C; relative humidity, 50–60%; and photoperiod of 16 h:8 h L:D) inside glass vials (about 15 cm length and 2.5 cm diameter) containing a meridic medium. The artificial diet was composed of a mixture of corn meal and brewers' yeast, added to 70% ethanol, agar and methylparaben as a fungistatic. Ten to fifteen 2-day old *D. melanogaster* females were placed in a glass vial with fresh medium, and were allowed to oviposit for about 48 h. New adult flies were obtained 7–9 days after oviposition.

T. drosophilae were reared in glass vials containing 1- to 2-day old *D. melanogaster* puparia, exposed to about 10–15 2-day old mated female parasitoids for about 48 h. The emergence of new parasitoids took place after 23–25 days.

Insect preparation for behavioural observations

Parasitized host puparia were collected 20 days after parasitization and isolated singly in small glass vials (5 cm length and 15 cm diameter) with a droplet of honey solution.

Newly emerged virgin parasitoid males and females (24–36 h old) were transferred from the incubator to room conditions (temperature 25–28°C, relative humidity 50%) for observation and alterations, i.e. ablation of both male and female antennae and glue applications (Supercolla Saratoga®, Milan, Italy) on the male antennae. In both cases, the parasitoids were anaesthetized in CO₂ and put on a cold table (Peltier effect, Labco®, Rome, Italy) kept at about 3°C. For the ablations, the antennae were positioned straight and all the antennomeres, except the scape (A1), were ablated using a scalpel blade under a stereomicroscope. The glue was applied using an insect pin (no. 000), taking care to restrict the glue only to the ventrolateral side of A4 or A5. After the alterations the parasitoids were replaced singly in a vial and used the following day.

The following bioassays were carried out: (a) intact males and females; (b) males with A4 covered with glue and intact females; (c) males with A5 covered with glue and intact females; (d) males and females with the antennae of the same side ablated, i.e. males and females without the right (or left) antenna; and (e) males and females with the antennae of the opposite side ablated, i.e. males without the right antenna and females without the left (or vice versa). The pairs were observed continuously for 10 min, starting from the first encounter between male and female. Ten to fourteen replicates for each bioassay were carried out. In the case of successful courtship (i.e. the female exposes its genital aperture to the male and the male moves rearwards to mate) the bioassays were terminated.

In the case of males with one antennomere covered with glue, after the bioassay each individual was isolated and processed for scanning electron microscope (SEM) observation, to check whether the glue was properly applied (Fig. 1E).

For the observations we used a VCR Panasonic NV-FS100HQ (Panasonic®, Osaka, Japan) connected to a video camera JVC KY-M280 (Panasonic®) mounted on a WILD M420 stereomicroscope

(Wild, Heerbrugg, Switzerland), and data were collected using the software The Observer Video Pro 4.0 for Windows® (Noldus Information Technologies, Wageningen, The Netherlands). In all bioassays, a virgin male and female were placed inside a PVC arena (15 mm diameter and 3.5 mm depth) covered with a coverslip.

Statistics

The behavioural parameters, and male antennation frequency and duration, were analysed using Kruskal–Wallis one-way analysis of variance by ranks. If the value of *H* was significant, comparisons were conducted using the Bonferroni–Dunn method. According to this method the familywise type I error is controlled under a non-directional hypothesis (Sheskin, 2004).

SEM observations

All the *T. drosophilae* males with glue applied to either A4 or A5 were kept separately and processed for SEM observation to check whether the glue had been correctly applied. In the case of glued A4 we checked whether the glue was restricted to the release site of the A4 gland, while in the case of glued A5 we checked whether the glue was applied only on A5. In all cases, anaesthetized males were beheaded and air dried, avoiding the use of any chemical to prevent the removal of the glue. After dehydration, the heads with antennae were gold coated in a Balzers Union SCD 040 unit (Balzers®, Vaduz, Luxembourg), and examined with a Philips XL 30 (FEI® Company, Eindhoven, The Netherlands).

RESULTS

In *T. drosophilae* the geniculate male antennae consist of 14 antennomeres, numbered from the scape (A1) to the apical antennomere (A14; Fig. 1A). A4 shows a cuticular carina on the ventrolateral side (Fig. 1B). Several cuticular pores were observed, either clean (Fig. 1D) or filled with secretion (Fig. 1C). A detailed morphological and histological study of this structure has been carried out by Sacchetti and colleagues (Sacchetti et al., 1999).

Mating behaviour

Observations with intact *T. drosophilae* individuals were conducted to define sequential mating steps.

Wing fanning. When a male perceived a female, a vigorous and continuous wing fanning was performed, without physical contact. Then the male followed and reached the female, keeping his wings vibrating and the antennae straight and forward.

Encounter. The male touched the female with the antennae.

Mounting. The male mounted the female with the two individuals facing the same way. The female raised the antennae quickly.

Antennation. The male brought his antennae in front of those of the female, which were kept upright and parallel (Fig. 2A). Then the male started to gently stroke the distal region of the female antennae (i.e. the club-shaped portion) with the ventrolateral region of the proximal part of his antennae (i.e. A4). The male often insistently stroked the apical part of the female's antennae (Fig. 2B). The contact lasted 43.71±4.40 s (mean ± s.e.m., *N*=14; Table 1) without interval. In one case only, the antennation was performed twice (antennation frequency 1.07±0.07, mean ± s.e.m., Table 1). Sexually receptive females reacted by extruding the ovipositor and exposing the genital opening, while their antennae were placed laterally and backwards. This change in the female's antennae position is likely to be the signal that stops the antennation by the male.

Copulation. The male quickly moved rearward, spread his wings and eventually copulated.

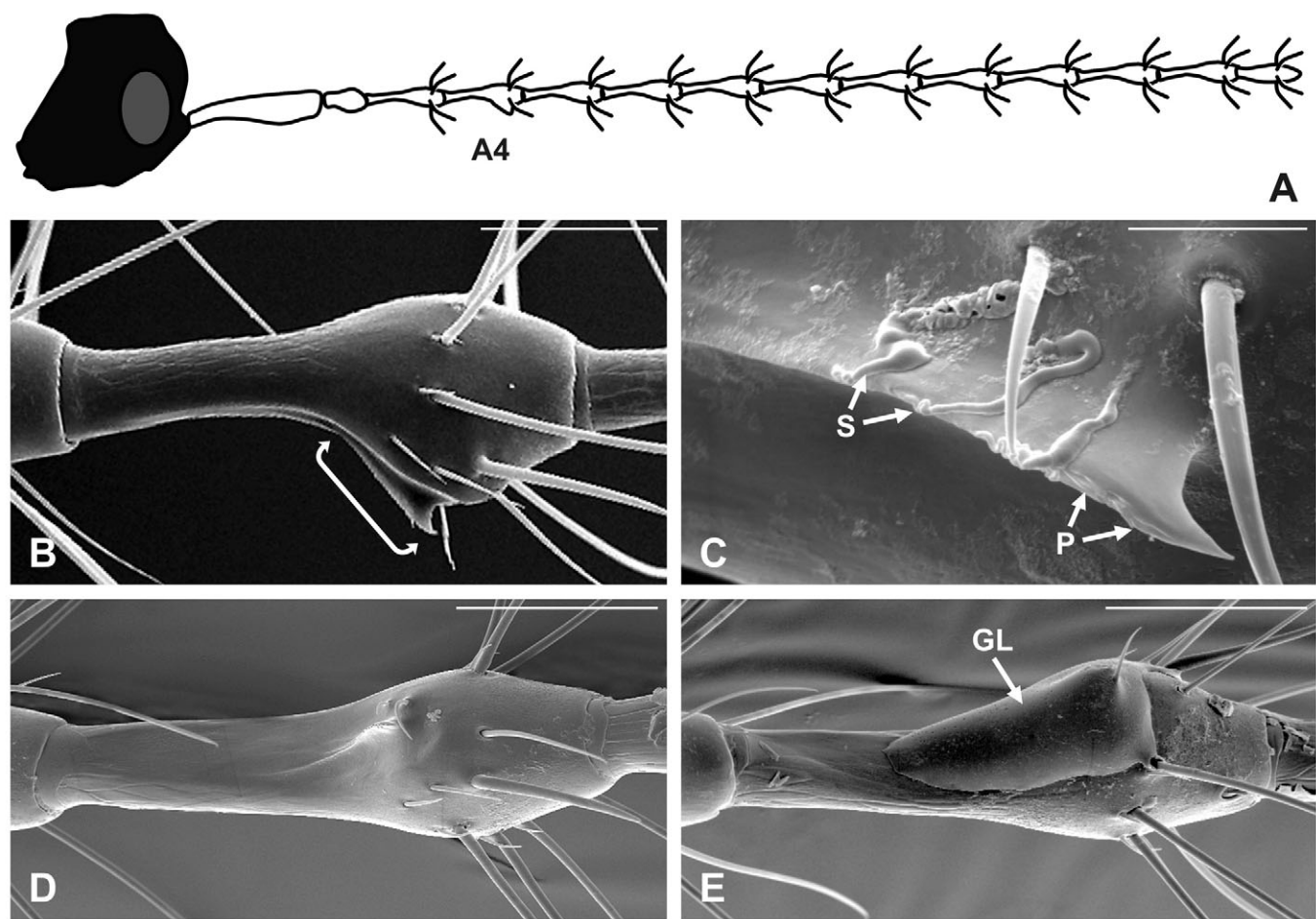


Fig. 1. (A) Schematic drawing of *T. drosophila* male antenna. (B–E) Scanning electron micrographs of *T. drosophila* male fourth antennomere (A4) showing the ventrolateral carina (in B), pores (P) and secretion (S) from the carina edge (in C), and the comparison between a clean and a glued (GL) fourth antennomere (D and E, respectively). Scale bar: B, D and E, 50 µm; C, 10 µm.

The courtship behaviour of *T. drosophila* is available as a video (see Movie 1 in supplementary material).

Bioassays

To understand the role of antennal glands, the following bioassays were performed with altered pairs. Results and statistical evidence are reported in Table 1.

Males with A4 covered with glue and intact females

These bioassays were performed to assess the role of the male antennal gland secretion (Fig. 3B). In this situation, the behavioural sequence followed the above described scheme for intact pairs, but

no copulation occurred ($N=12$). After several copulation attempts, the male either gave up or was made to dismount by the female. The antennal contact lasted 125.55 ± 20.59 s (mean \pm s.e.m.), with an antennation frequency of 2.83 ± 0.66 (mean \pm s.e.m.), both values significantly higher than for the intact pairs.

Males with A5 covered with glue and intact females

These bioassays were performed to assess possible side effects of the glue. The behavioural sequence was similar to that described for intact pairs, leading to 100% of matings ($N=10$). The antennation time (50.69 ± 7.15 s, mean \pm s.e.m.) was not significantly different from that of intact individuals, but was significantly shorter than

Table 1. Effect of antennal manipulation on mating in *T. drosophila*

Pairs	N	Mating frequency (%)	Mean (\pm s.e.m.) no. of antennation attempts	Mean (\pm s.e.m.) antennation time (s)
Intact male and female	14	100	1.07 \pm 0.07 ^a	43.71 \pm 4.40 ^a
Male with glued A4 and intact female	12	0	2.83 \pm 0.66 ^b	125.55 \pm 20.59 ^c
Male with glued A5 and intact female	10	100	1.20 \pm 0.20 ^a	50.69 \pm 7.15 ^{a,b}
Male and female with antennae ablated on the opposite side	10	0	3.90 \pm 0.96 ^b	120.89 \pm 26.71 ^c
Male and female with antennae ablated on the same side	10	100	1.20 \pm 0.13 ^a	56.23 \pm 7.42 ^b

Kruskal–Wallis test, d.f.=4, $N=56$: mean no. of antennation attempts: $H=21.99$, $P=0.0002$; mean antennation time (s): $H=21.35$, $P=0.0003$. Different superscript letters indicate significant differences between treatments (Bonferroni–Dunn test).

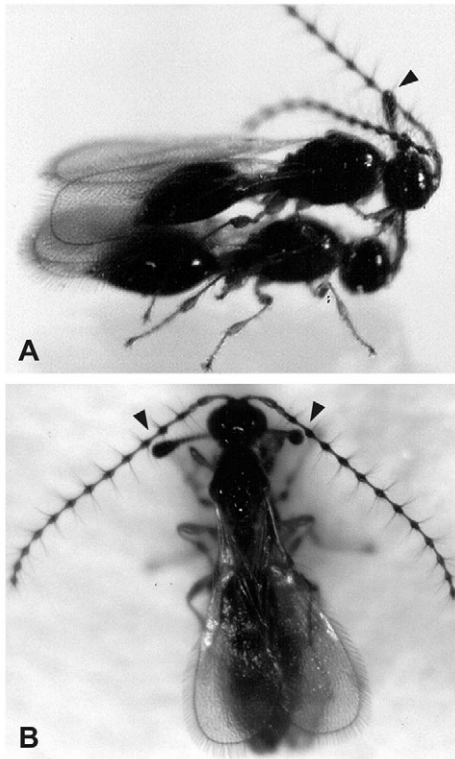


Fig. 2. Courtship positions in *T. drosophilae*. (A) Lateral view of a pair showing the male (top) and the female (bottom). (B) Dorsal view of a pair. Arrowheads indicate the antennal contact between male A4 and female apical antennomeres.

that for males with A4 covered with glue. A second antennation attempt was recorded only in one case (antennation frequency 1.20 ± 0.20 , mean \pm s.e.m.).

Males and females with antennae of the opposite side ablated. These bioassays were performed to assess whether the gland secretion acts on contact (Fig. 3D). After mounting, the male performed the antennation, with no contact with the female's opposite antenna: no copulation occurred ($N=10$). The antennation time lasted 120.89 ± 26.71 s (mean \pm s.e.m., $N=10$), with an antennation frequency of 3.90 ± 0.96 (mean \pm s.e.m.), both significantly higher than for intact pairs and pairs with males with glue on A5, but not different from males with glue on A4. Males tried to copulate unsuccessfully and/or were made to dismount by females, which did not show the typical signals of receptivity.

Males and females with the antennae of the same side ablated. These bioassays were performed to assess possible side effects of antennal ablation (Fig. 3C). After mounting, the male started antennating the female for a longer time, when compared with intact pairs (56.23 ± 7.42 s, mean \pm s.e.m., $N=10$). However, antennal contact was not different from pairs with males with glue on A5. Copulation occurred in 100% of bioassays.

DISCUSSION

In *T. drosophilae* antennae play a fundamental role in mediating courtship behaviour. In this species, mating behaviour occurs according to a stereotyped sequence, in which antennal contact between individuals during courtship is essential. During our experiments, we were able to prevent the transfer of the male sex recognition pheromone covering the release site of the glanded

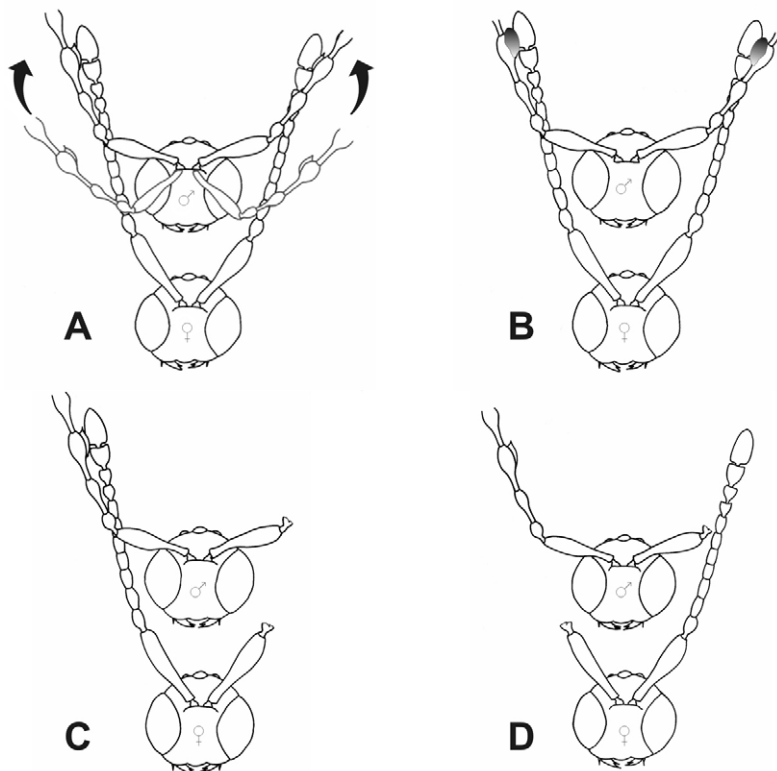


Fig. 3. Schematic drawing illustrating the relative position of male and female antennae during courtship. (A) Intact pairs; (B) males with glue applied on A4; (C) pairs with antennae ablated on the same side; (D) pairs with antennae ablated on opposite sides.

antennomere using a strong glue. The presence of a single glanded antennomere on the male antenna made it possible to carry out these experiments, through which we have demonstrated that antennal contact is necessary for mating.

This was further demonstrated by selective ablation bioassays. In cross-ablated individuals we were able to prevent mating, while this occurred normally in the case of same-side ablated pairs. These facts support our hypothesis that the courtship pheromone needs to be transferred from male to female antennae through contact, since the short distance between the antennae in cross-ablated individuals could allow the perception of a short-range volatile pheromone. Behavioural experiments also demonstrated that antennal contact is associated with an exact posture between male and female, according to a 'lock and key' mechanism allowing the transfer of the pheromone onto female antennal receptors.

Sacchetti and colleagues reported the occurrence of male antennal glands on the fourth antennomere of *T. drosophilae* males (Sacchetti et al., 1999). Ultrastructural details of the secretory cells as well as the physical features of the secretion (i.e. viscous appearance) are consistent with a possible contact activity of the secretion.

The pheromone-based courtship behaviour performed by *T. drosophilae* males could actually be an integration of different stimuli provided for the female, i.e. contact and visual in combination with chemical. Although we did not investigate the role of visual cues, mechanical stimulations were offered by the male throughout the experiments, except for the ones with cross-ablation of the antennae.

Antennal ablation has proved to be an effective tool in investigating insect behaviour related to the functional significance of a specific organ, as already reported for antennae in the cricket *Teleogryllus oceanicus* (Le Guillou) (Balakrishnan and Pollack, 1997) and the parasitic wasp *Leptopilina heterotoma* Meigen (Isidoro et al., 1999), and for the mechanosensilla of male genitalia in *Drosophila* (Acebes et al., 2003).

The presence of antennal glands in males is a common feature, shared by primitive and evolved hymenopterans. In fact, males of Terebrantia (e.g. Bin and Vinson, 1986; Bin et al., 1999) and Aculeata (Felicoli et al., 1998; Isidoro et al., 2000; Romani et al., 2003; Romani et al., 2005) have glanded antennomeres.

The role of the secretion in sex recognition has been proved in several species of Terebrantia (Bin et al., 1988; Bin et al., 1999; Isidoro et al., 1999) and in one species of Aculeata (Felicoli et al., 1998).

Besides Hymenoptera, antennal glands have been described in a few other insect orders (Skilbeck and Anderson, 1994; Weis et al., 1999; Giglio et al., 2005; Belcari and Kozánek, 2006), as well as the presence of the male antennal glands associated with courtship behaviour (Medvedev and Pavlov, 1988; Bartlet et al., 1994).

Male glands involved in the production of courtship pheromones can be located on body parts other than the antennae, i.e. legs (Wittman and Blochtein, 1995). In Lepidoptera, male coremata, i.e. eversible scent brushes present on the abdomen, produce courtship pheromones (Haynes and Birch, 1985). The same function has also been reported for the wings of some butterflies (Grula et al., 1980; Honda, 1980).

The results we found in *T. drosophilae* are of great relevance for studies that attempt to interpret the mating system in Hymenoptera, one of the largest, biologically diverse and economically important insect orders (Ayasse et al., 2001). Large differences have been found so far in terms of number, distribution and cytological features of the antennal glands. These differences can be interpreted as a great diversity in anatomical features of

antennal structures, with a convergent functional significance. The hypothesis of the lock and key mechanism, i.e. the fact that the morphology of animal genitalia can be used as a highly species-specific tool (Shapiro and Porter, 1989), could be applied to antennal structures, at least in Hymenoptera Apocrita, the insect group that seems to rely more than others on the antennae during mating behaviour.

We thank one anonymous reviewer for valuable comments on an earlier version of the manuscript. We are grateful to Dr R. Allemand (Laboratoire de Biométrie, Génétique et Biologie des Populations, Université Claude Bernard Lyon I, Lyon, France) for providing the *T. drosophilae* and *D. melanogaster* colonies. SEM photographs were made at the Electron Microscopy Center, Perugia University. This research was financially supported by the MiUR (Ministry for University and Research, CoFin 'Reproductive strategies of parasitoid hymenoptera for biological control of crop insect pests').

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